

12

EUROPEAN PATENT APPLICATION

21 Application number: 88303657.6

22 Date of filing: 22.04.88

51 Int. Cl. 4: **C 12 Q 1/68**
C 12 P 21/02, C 12 N 15/00,
A 61 K 39/04

30 Priority: 24.04.87 GB 8709803

43 Date of publication of application:
26.10.88 Bulletin 88/43

84 Designated Contracting States: ES GR

71 Applicant: **McFadden, John-Jo**
Department of Surgery St. George's Hospital Medical
School Cranmer Terrace
London SW17 0RE (GB)

Hermon-Taylor, John
Department of Surgery St. George's Hospital Medical
School Cranmer Terrace
London SW17 0RE (GB)

72 Inventor: **McFadden, John-Jo**
Department of Surgery St. George's Hospital Medical
School Cranmer Terrace
London SW17 0RE (GB)

Hermon-Taylor, John
Department of Surgery St. George's Hospital Medical
School Cranmer Terrace
London SW17 0RE (GB)

74 Representative: **GoldIn, Douglas Michael et al**
J.A. KEMP & CO. 14, South Square Gray's Inn
London WC1R 5EU (GB)

The microorganism(s) has (have) been deposited with the National Collections of Industrial and Marine Bacteria under number(s) NCIB 12481.

54 **Diagnostics and vaccines for mycobacteria in public health, medical and veterinary practice.**

57 The invention relates to a family of DNA insertion sequences (ISM) of mycobacterial origin and other DNA probes which may be used as probes in assay methods for the identification of mycobacteria and the differentiation between closely related mycobacterial strains and species. In one method the probes are used to distinguish pathogenic M. paratuberculosis from M. avium, which finds an application in the diagnosis of Crohn's disease in humans and Johne's disease in animals. The use of ISM, and of proteins and peptides encoded by ISM, in vaccines, pharmaceutical preparations and diagnostic test kits is also disclosed.

BEST AVAILABLE COPY

Description

DIAGNOSTICS AND VACCINES FOR MYCOBACTERIA IN PUBLIC HEALTH, MEDICAL, AND VETERINARY PRACTISE

INTRODUCTION.

It is estimated that 3 to 6 million people in the world die each year from leprosy and tuberculosis alone. Human disease due to atypical mycobacteria is increasing. Tuberculosis and paratuberculosis are common in livestock, account for major economic losses, and form a reservoir of infection for human disease. Despite BCG vaccination and some effective drugs, mycobacterial disease remains a major global problem.

Present methods for the identification and characterisation of mycobacteria in samples from human and animal diseases are by Zeil-Neelson staining, in-vitro and in vivo culture, biochemical testing and serological typing [1]. These methods are generally slow and do not readily discriminate between closely related mycobacterial strains and species particularly, for example, Mycobacterium paratuberculosis and Mycobacterium avium. Mycobacteria are widespread in the environment, and rapid methods do not exist for the identification of specific pathogenic strains from amongst the many environmental strains which are generally non-pathogenic. Difficulties with existing methods of mycobacterial identification and characterisation have increased relevance for the analysis of microbial isolates from Crohn's disease (Regional Ileitis) in humans [2] and Johne's disease in animals [3] (particularly cattle, sheep and goats) as well as for M. avium strains from AIDS patients with mycobacterial superinfections [4]. Although recognition of the causative agents of human leprosy and tuberculosis are clear, clinico-pathological forms of each disease exist, such as the tuberculoid form of leprosy, in which mycobacterial tissue abundance is low and identification correspondingly difficult. Improvements in the specific recognition and characterisation of mycobacteria may also increase in relevance if current evidence linking diseases such as rheumatoid arthritis to mycobacterial antigens is substantiated [5]. Emerging drugs resistance to mycobacteria including M. avium isolates from AIDS patients, and Mycobacterium tuberculosis from TB patients is an increasing problem. Rapid methods for the identification of mycobacteria in environmental and disease samples, for the specific recognition of pathogenic strains, for precisely distinguishing closely related mycobacterial strains, and for defining resistance patterns to antimycobacterial agents are badly needed.

THE INVENTION

1. DNA probes for the specific identification of mycobacteria

A method of using DNA probes for the precise identification of mycobacteria and discrimination between closely related mycobacterial strains and species by genotype characterisation. The method

of genotypic analysis is further applicable to the rapid identification of phenotypic properties such as drug resistance and pathogenicity.

DNA probes of the invention are derived from recombinant DNA libraries or synthesised using DNA sequence information from recombinant DNA clones derived from different mycobacterial strains and species. DNA clones are selected for their ability specifically to identify mycobacterial DNA from other microbial DNA. DNA probes of the invention are also selected for their ability to distinguish and differentiate between closely related mycobacterial strains and species such as Mycobacterium paratuberculosis and Mycobacterium avium, and different strains of mycobacteria including those isolated from AIDS patients. DNA probes of the method of the invention are also selected for their ability to identify and predict mycobacterial phenotypes, including drug resistance, by genotype characterisation.

2. Diagnostics and vaccines for public health, medical and veterinary use based upon Mycobacterial Insertion Sequences (ISM).

We have identified, for the first time, a family of related DNA insertion sequences (ISM) in the genome of disease isolates of mycobacteria. The first example of ISM (ISM-1) we identified and characterised in the clone pMB22 (Deposited with the National Collections of Industrial and Marine Bacteria, NCIB number 12461, Aberdeen, Scotland) from a genomic DNA library derived from a mycobacterial isolate from Crohn's disease (CD). We also identify ISM-1 in Mycobacterium paratuberculosis cultured from cattle and other animals suffering from Johne's disease (JD). We identify related sequences of the ISM family in mycobacterial disease isolates superinfecting humans with AIDS (acquired immune deficiency syndrome), atypical mycobacterial pneumonias in immunocompetent humans, and other mycobacterial disease isolates from animals and birds. We also identify ISM in isolates of Mycobacterium leprae and Mycobacterium lepraemurium. ISM sequences appear to be prevalent in pathogenic mycobacteria, but have not been identified in saprophytic mycobacteria.

All or part of the DNA sequence of each member of the ISM family may be used with or without enzymic amplification by polymerase chain reaction (PCR) or other techniques, as DNA or RNA probes in diagnostics for the specific recognition of pathogenic mycobacteria in environmental and disease samples and in in-vitro mycobacterial cultures. All or part of the proteins or peptides encoded, mutated or modified in expression by ISM may be used in immunodiagnostics or other diagnostics for a similar purpose whether based on recombinant proteins or synthetic peptides. Such ISM derived proteins or peptides may also be used as specific vaccines for the prevention of mycobacterial disease in medical and veterinary practise. Alteration of microbial

phenotype by deletion or transfection of ISM in whole or in part may be used to engineer culturable microbial strains suitable for use as vaccines, in immunotherapy or diagnostics.

SUMMARY OF THE FIGURES:

Figure 1 shows the multiple banding pattern or DNA "fingerprint" obtained when restricted genomic DNA from each of three separate Crohn's disease (CD) mycobacterial isolates and one Johne's disease (JD) *M. paratuberculosis* isolate are probed with pMB22 containing ISM-1 of the invention. Figure 1 also shows a different and much simpler banding pattern with restricted genomic DNA from 4 other species of mycobacteria which do not contain ISM-1 or homologous insertion sequences conferring pathogenicity. This figure illustrates how a shared and complex banding pattern can demonstrate identity between mycobacteria as between CD and JD isolates, as well as distinguish precisely between different mycobacteria.

Figure 2 supports Figure 1 by showing identity between further independent CD mycobacterial isolates from Amsterdam, Holland and Los Angeles, U.S.A. both being different from *M. avium*. Figure 3 shows a restriction map which characterises the 5 kb of insert DNA containing the sequence ISM-1 of the invention, in the clone pMB22. Fig 3 also shows mapping data obtained from *M. avium* and *M. paratuberculosis* DNA indicating that DNA flanking ISM-1 in *M. paratuberculosis* is identical to DNA found at the same site in *M. avium* but without of course, an associated ISM-1. This indicates that ISM-1 is inserted into the genome of *M. paratuberculosis* at this site and is a likely cause of the divergent phenotype. Figure 4 further characterises the insert cloned in pMB22 with partial DNA sequence data spanning the ISM-1 insertion element. Figure 5 shows the multiple banding patterns obtained from a series of pathogenic strains of *M. avium* which are shown to contain other ISM sequences related to the insertion sequence ISM-1 found in pMB22. These related ISM sequences may be involved in pathogenicity of these and other mycobacteria, particularly those of the *M. avium* complex. Figure 5 also demonstrates the use of DNA fingerprinting to identify further pathogenic mycobacteria and distinguish them from environmental mycobacteria.

THE FOLLOWING EXAMPLES ILLUSTRATE THE INVENTION

Example 1. DNA probes for the specific identification of mycobacteria.

DNA probes were derived as follows. Genomic DNA from a strain of *M. paratuberculosis* isolated from a human CD patient [6] was digested with Bam H1, ligated into the plasmid vector pGEM-1 (Promega-Biotech), and used to transform *E. coli* DH 1 cells using standard techniques (Maniatis et al. 1982. Molecular Cloning: Laboratory Manual). Greater than 20,000 ampicillin resistant recombinant clones were obtained. Insert size in plasmid DNA from 12 randomly selected clones (pMB1 to 12) ranged from

180bp to 4200 bp. This library was screened by hybridisation with hexanucleotide primed, radiolabelled total genomic DNA probes from *M. paratuberculosis* strain Ben, *M. paratuberculosis* (ATCC 19698), and *M. kansasii* (TMC 1201) Hybridising colonies were identified by autoradiography. Approximately 5% of clones hybridised to both *M. paratuberculosis* (ATCC 19698) and *M. paratuberculosis* (strain Ben) and 1% to *M. kansasii*. Forty eight clones were selected as giving either a differential hybridisation signal with DNA from the three sources, or a similar signal with DNA from the three sources, and gridded onto an ordered array. The ordered array was again hybridised with radiolabelled genomic DNA from *M. paratuberculosis* strain Ben, *M. paratuberculosis* (ATCC 19698), *M. avium* complex serotype 2 (Caddigg 16741), *M. avium* complex serotype 5 (25546-759) *M. kansasii* (TMC 1201) and *M. phlei* (NCIB 8573). Clones designated pMB13 to 24 were selected as showing either strong hybridisation to all DNA samples, or demonstrating differential hybridisation between the DNA samples.

Example 2 - DNA probes for 16s ribosomal RNA

Probes encoding ribosomal RNA sequences were isolated by screening the *M. paratuberculosis* strain Ben genomic library with radiolabelled complementary DNA (cDNA) prepared from total RNA extracted from *M. avium* serotype 2 cells. Cells were lysed by sonication for 80 seconds in 4M guanidinium isothiocyanate and RNA purified by centrifugation through 5.7M caesium chloride and precipitated with 70% ethanol. Radiolabelled cDNA was prepared by incubating 1µg of purified RNA with reverse transcriptase, using random hexanucleotides to prime cDNA synthesis. The radiolabelled probe was hybridised to the bacterial colonies *in situ*. Autoradiography revealed hybridising clones which were picked and plasmid DNA extracted. This was radiolabelled and used to probe Northern blots of *M. avium* total RNA. A clone designated pMB16 was found to hybridise strongly to 16S ribosomal RNA on Northern analysis.

Example 3 - DNA probes for *M. bovis* and *M. Leprae*

In a further example of the preparation and use of DNA probes for the recognition and differentiation of a specific mycobacterial strain, DNA from *Mycobacterium bovis* (BCG) strain Glaxo was digested with Bam H1, ligated to vector pGEM-1 and used to transform *E. coli* DH1 cells by standard techniques as before. The resulting genomic DNA library was probed with radiolabelled *M. bovis* genomic DNA and strongly hybridising clones designated pMBCG13-24 picked. Similarly, genomic DNA was extracted from two nude mouse isolates of *Mycobacterium leprae* obtained from lepromatous leprosy patient tissue and a genomic library of several thousand clones prepared. Twelve randomly selected clones containing inserts and designated pML1-12 were isolated.

Example 4 - The use of mycobacterial DNA probes

The precise identification and differentiation between mycobacterial strains and species is in general

performed by the isolation of genomic DNA from target bacteria, digestion with restriction endonucleases, electrophoresis, and Southern blotting using one of the mycobacterial DNA probes radiolabelled as described. Autoradiography reveals banding patterns that are examined for restriction fragment length polymorphisms (RFLPs) but dot blot analyses and non radioactive revealing systems as well as PCR amplification may also be employed. RFLPs are also used to measure DNA base substitution between strains and species.

Differentiation between *M. paratuberculosis* and *M. avium* complex. DNA from *M. paratuberculosis* ATCC 19698, *M. paratuberculosis* strain Ben, and *M. avium* serotype 2 (Caddig 16741), was digested with restriction endonucleases Bam H1, EcoR1, Bst 1, Ava 1, pst 1, Hinf 1, Hae III, Sau 3A, Hinc 1, Pvu 11, Pvu 1 and Taq 1, electrophoresed, blotted and probed with radiolabelled clones, pMB7, pMB12, pMB16, pMB17, pMB19, pMB20, pMB21, pMB22, and examined for RFLPs that distinguished between the DNA samples. (The derivation of clone pMB22 which contains the insertion sequence ISM-1 of the invention, is described in example 5). Several RFLPs were found to distinguish between the *M. avium* and the *M. paratuberculosis* strains [7]. However, no RFLPs were found to distinguish between *M. paratuberculosis* ATCC 19698 and *M. paratuberculosis* strain Ben including the complex banding pattern seen with pMB22 containing the ISM-1 insertion sequence [7], suggesting genetic identity between these two. The vaccine strain 18 presumed to be of *M. paratuberculosis* was further shown in fact to be identical to *M. avium* serotype 1 [8].

Identification of Crohn's disease (CD) derived mycobacteria. Several isolates of mycobacteria from CD tissue have been thought to be related to *M. paratuberculosis* [6,9]. However, conventional techniques, which are incapable of distinguishing precisely between *M. paratuberculosis* and *M. avium* (particularly mycobactin-dependent *M. avium*) have left the identity of these CD isolates uncertain. Since *M. avium* complex strains can be isolated from healthy subjects, and are common environmental organisms [10], the exact identity of the CD-derived strains is critical to an evaluation of their etiological significance in CD. DNA was therefore extracted from three CD-derived strains (Ben, Linda and Dominic) [6], and also *M. avium* complex serotype 2, *M. avium* complex serotype 5, *M. kansasii* and *m. phlei*. DNA was digested with restriction endonucleases and probed with DNA clones of the invention identifying RFLPs as described that differentiated between *M. paratuberculosis* and *M. avium* complex serotype 2. Six different RFLPs were investigated. Each of the three CD-derived strains were shown to be identical to *M. paratuberculosis* [7] (Fig 1). These findings were supported by the use of the ISM-1 containing clone pMB22 of the invention to probe restricted DNA from two further uncharacterised human CD mycobacterial isolates, 410 & H1 obtained from two independent laboratories, of Drs Gitnik and Beaman UCLA in the U.S.A and Dr. J. Haagsma, National Veterinary Institute, Amsterdam, Netherlands respectively. Identical banding patterns

were obtained showing that these further CD isolates were again ISM-1 containing *M. paratuberculosis*. (Fig 2).

Similar methods and DNA probes of the invention were used to examine the identity of 8 strains of *M. paratuberculosis* isolated from infected cows with Johne's disease. Isolates were derived independently from infected animals in the UK, France and the U.S.A., as well as 2 additional isolates from Macaque monkeys suffering from a disease resembling Johne's disease [11]. RFLP analysis with probes pMB14, pMB20, and pMB22 revealed that all strains gave identical banding patterns to those seen in Figs 1 and 2 for pMB22, demonstrating the conserved nature of the pathogen *M. paratuberculosis*. Probing with clone pMB22 containing insertion sequence ISM-1, revealed identical banding patterns for each strain.

Members of the *M. avium* complex of mycobacteria have been difficult to characterise and define by conventional techniques. We used the mycobacterial probes and methods of the invention described, to examine a range of *M. avium* complex strains. Several different patterns were obtained [8]. Serotypes 2,4,5,6, and 8 were found to be similar with less than 20% DNA base substitution between strain types. However, at least 6 strain type banding patterns were recognised. *M. avium* complex serotypes 11, 16 and 27 were found to have greater than 13% DNA base substitution between each other and the serotype 2,4,5,6,8 group. These strains were previously designated *M. intracellulare* or intermediate strains. We proposed from this work that *M. avium* complex strains giving banding patterns similar to those obtained with serotypes 2,4,5,6 and 8 to be designated *M. avium* and the heterogeneous strains represented by serotypes 11, 16 and 27 be designated *M. intracellulare* complex [8]. Strain typing using the higher resolution DNA probe methods of the invention did not always correspond with conventional serotyping.

The methods and DNA probes of the invention were further used to distinguish *M. bovis* BCG (Glaxo) and *M. tuberculosis* strain H37RV. DNA from these mycobacteria was digested with Ava 1, Sma 1, Kpn 1, Pvu II and Pst 1, electrophoresed, blotted and probed with pMB16 and pMBCG13. pMB16 demonstrated an RFLP between Kpn 1 digests of *M. bovis* and *M. tuberculosis* DNA and pMBCG13 revealed an RFLP with Sma 1 digested DNA.

Similar techniques were applied to the characterisation of *M. leprae*. *M. leprae* DNA was cut with Pvu II electrophoresed, blotted and probed with pMB16 and pML1. pML1 gave a banding pattern with *M. leprae* DNA but did not hybridise to DNA from other mycobacteria. pMB16 gave an *M. leprae* banding pattern distinct from those seen with any other mycobacteria tested.

DNA was extracted from a range of mycobacteria, digested with Pvu II, electrophoresed, blotted, and probed with radiolabelled clone pMB16 and other clones. The results demonstrated that each mycobacterial species gave a distinct and identifiable DNA banding pattern.

The use of cloned DNA probes of the invention for

the specific identification of mycobacteria is also applicable to DNA extracts of tissues infected in vivo with mycobacteria. To demonstrate this, we extracted mouse liver infected with mycobacterium lepraemurium using methods we developed to extract mycobacterial DNA [8]. Extracted DNA was cut with Pvu II electrophoresed, blotted and probed with pMB12, pMB16, pMB20 and pMB22. Specific banding patterns were obtained for M. lepraemurium from mouse liver which showed this organism to be closely related to M. avium having an approximate 20% base substitution between it and M. avium complex serotype 2 [8].

Example 5. The Identification, cloning and characterisation of mycobacterial insertion sequences (ISM) of the invention.

Insertion sequences and transposons are short repetitive pieces of DNA within genomic DNA, which may be mobile in response to appropriate environmental conditions. They exert their biological effects either through the components which they encode or by their influence on neighbouring host genes, or host genes into which they become inserted. These elements occur widely in nature and have been identified in some bacteria, yeasts, plants, insects and animals [12]. They have not hitherto been identified in mycobacteria.

A major aspect of the present invention comprises the identification, for the first time, of a novel family of related insertion sequences in disease isolates of mycobacteria. This family of insertion sequences is designated ISM, and an example of ISM (ISM-1) we have cloned and characterised in detail. Members of the ISM family of mycobacterial insertion sequences of the invention are identified by sharing partial sequence homology and hybridising at low stringency with ISM-1 in the clone pMB22 deposited with the National Collections of Industrial and Marine Bacteria, number 12461 Aberdeen, Scotland, U.K. All or part of the DNA or RNA encoded by members of the ISM family and all or part of the proteins and peptides encoded by ISM or mutagenised or altered in expression by ISM are applicable to use in a novel range of diagnostics for disease-causing mycobacteria containing ISM. ISM of the invention are also used by specific deletion or transfection, to generate mutant or recombinant bacteria or viruses for the provision of novel vaccines, agents for immunotherapy, or diagnostics.

Genomic DNA from a Crohn's disease isolate of Mycobacterium paratuberculosis was used to generate a genomic DNA recombinant library using the plasmid vector pGEM-1 and E. coli DH1 cells by standard techniques as described. This library was screened with radiolabelled DNA probes derived from M. paratuberculosis, M. kansasii and M. avium. There was only one clone identified which hybridised strongly to M. paratuberculosis but only weakly to M. avium and M. kansasii DNA and we designated this clone pMB22. This was picked and found to contain as insert of about 5kb. This recombinant DNA fragment was excised, purified, digested with further restriction endonucleases, and sub-cloned into either the plasmid vector pGEM-1, or the M13 phage

vectors mp18 and mp19. Resulting clones were hybridised to M. paratuberculosis DNA and strongly positive clones were identified. The location of selected subclones, within the 5kb insert in pMB22, some of which hybridised strongly with M. paratuberculosis DNA, are shown (Fig 3A). E. coli strain DH1 harbouring pMB22 was deposited on 24th April, 1987 under Budapest treaty conditions, with the National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, U.K. where it was given the deposit number NCIB 12461.

Example 6. Insertion sequence ISM-1 in M. paratuberculosis.

This section cites evidence showing that ISM-1 in M. paratuberculosis are insertion sequences and that these segregate with the pathogenicity of this organism.

M. paratuberculosis DNA was cut with Bam H1, Sst1, EcoR1 and Bst1, electrophoresed, blotted, and hybridised to pMB22 and its subclones pMB22/S4 and pMB22/S12. In each case approximately 8 strongly hybridising bands were obtained. When this procedure was repeated using the restriction endonucleases Alu1, Ava1, Pst1 PvuII and Pvu1, approximately 16 strongly hybridising bands were seen with pMB22 and its subclone S4, while a subset of only 8 of these bands hybridised to the subclone of pMB22, S12. Digestion of M. paratuberculosis genomic DNA with Hinf1 produced 2 bands hybridising strongly with pMB22 and pMB22/S4, only one of which also hybridised with pMB22/S12. pMB22 and its subclones S4 and S12 were then used to probe Southern blots of Bam H1 restricted genomic DNA obtained from two laboratory strains of M. avium serotype 2 (Caddigg 16741) and serotype 5 (25546-759) [7]. A single band was found with both M. avium strains using pMB22 probe. pMB22/S12 did not hybridise to M. avium serotype 2 DNA, and pMB22/S4 hybridised only very weakly to a single band.

These results, taken together with those in example 5, indicate that pMB22 contains a sequence that is present in approximately 8 copies dispersed in the genome of M. paratuberculosis but absent in M. avium serotype 2. The enzymes giving 8 hybridising bands must not cut the insertion sequence contained within clone pMB22 and each band therefore represents a different genomic location of the ISM insertion elements. Enzymes such as PvuII cut the element once and therefore produce two fragments for each genomic copy. The sequence pMB22/S12 must be derived from one of the Pvu II fragments, whereas pMB22/S4 spans the PvuII site. The enzyme Hinf 1 must have at least 3 sites within the insertion element, and therefore each genomic copy of ISM is cleaved to give the same two internal ISM fragments, plus heterogenous ISM fragments containing flanking DNA. A detailed restriction endonuclease map of pMB22 was derived and is shown (Fig 3A).

The data presented show that the M. paratuberculosis and M. avium complex strains tested are very closely related, exhibiting less than 1% DNA base substitution between the two species [7,8]. How-

ever, the identical nature of *M. paratuberculosis* strains isolated from a wide variety of geographical sources and disease hosts, suggests that *M. paratuberculosis* is a conserved specific pathogen causing Johne's disease in cattle, goats, and at least one species of primate, and possibly causing some cases of Crohn's disease in humans. This would suggest there are specific genetic changes that characterise *M. paratuberculosis*, conferring pathogenicity and enabling it to cause these diseases. The tight segregation of ISM-1 insertion sequences to *M. paratuberculosis* and the specific absence of ISM-1 from most laboratory strains of *M. avium* examined, would make ISM-1 a strong candidate sequence responsible for the genetic changes in *M. paratuberculosis* endowing its pathogenicity.

The organisation of genomic DNA flanking the insertion sequence ISM-1 of the invention, identified within clone pMB22 and *M. paratuberculosis*, was examined with pMB22 and subclones, S4, S8, and S12 and the results compared with similar studies on *M. avium* genomic DNA. The maps are shown (Fig. 3B). Genomic DNA flanking ISM-1 in pMB22 showed no changes detectable between *M. avium* and *M. paratuberculosis*, indicating a similar level of base substitution between DNA in this region as elsewhere in the genome. Since *M. paratuberculosis* and *M. avium* are evolutionarily very close, the presence of the repetitive element ISM-1 in *M. paratuberculosis* is compatible with ISM-1 being recently acquired by *M. paratuberculosis* causing a divergence from *M. avium*. Alternatively the sequence ISM-1 may have been deleted from *M. avium* during evolution causing its divergence from *M. paratuberculosis*. Since the latter proposal would require precise excision and deletion of each copy of ISM-1 from the *M. paratuberculosis* genome, an extremely unlikely event, the former explanation is more plausible. The repetitive sequence ISM-1 would form an example of the genetic elements known as Insertion Sequences [12]. The DNA sequence of ISM-1 was determined by single-stranded M13 sequencing and is shown (Fig 4).

Example 7. ISM related sequences in other mycobacteria.

This section demonstrates the presence of sequences in other pathogenic mycobacteria which are related to ISM-1 by sharing homology, and which are identified by hybridisation with pMB22 or its subclones under appropriate experimental conditions. As with ISM-1, other members of the ISM family appear to confer pathogenicity on the mycobacterial host.

DNA from a collection of mycobactin-dependent *M. avium* strains isolated from diseases in wood-pigeon, deer, goat, pig and armadillo and also *M. avium* serotype 2 and *M. paratuberculosis* were hybridised with pMB22 and its subclones. Hybridisation of mycobactin-dependent *M. avium* strains with pMB22 gave banding patterns similar to those obtained with DNA from *M. avium* serotype 2, but also showed a series of additional bands, not present in *M. avium* serotype 2 (Fig 5A). When washed at high stringency the signal from most of

these additional bands was selectively removed. When representative DNA samples were probed with subclone pMB22/S4, these additional bands hybridised strongly, but only one faint band was obtained with *M. avium* serotype 2, derived from flanking DNA (Fig 5B). The subclone pMB22/S12 hybridised ISM-1 in *M. paratuberculosis* DNA but only to a single band in samples 157R and 157S, armadillo-derived mycobactin-dependent strains (Fig 5C). The non-mycobactin dependent armadillo-derived strain of *M. paratuberculosis* 10911 did not hybridise either to pMB22/S4 or pMB22/S12. When these DNA samples were probed with an unrelated *M. paratuberculosis* DNA probe, very similar or identical banding patterns were obtained from all strains.

These results indicate that pathogenic mycobactin-dependent strains of *M. avium* contain insertion sequences related to ISM-1 in the pathogen *M. paratuberculosis*. The strains 157R and 157S contain an insertion sequence closely related to ISM-1 since it hybridised to both pMB22/S4 and pMB22/S12. The insertion sequence present in the deer, goat, wood pigeon and porcine mycobacterial isolates are less closely related to ISM-1 and do not contain sequences homologous to pMB22/S12. The banding patterns were similar for some of the different strains in this group and the insertion sequence in these strains has been designated ISM-2.

Further unselected *M. avium* strains were investigated for the presence of insertion sequences of the ISM family. Several strains were found to contain copies of ISM-2 or related insertion sequences, but to date no other mycobacterial strains containing ISM-1 have been identified. Two further pathogens have been found to contain ISM-related insertion sequences. DNA from the leprosy bacillus *M. leprae* contains a sequence that hybridised to pMB22/S4. DNA from the rat leprosy bacillus *Mycobacterium lepraemurium* contained a sequence that hybridised to pMB22/S4, but not to pMB22/S12.

These results show that insertion sequences related to ISM-1 are found in a number of mycobacterial pathogens, particularly within the *M. avium* complex including those isolated from AIDS patients. We have screened more than 20 further mainly saprophytic and environmental mycobacterial species and strains and found none to contain insertion sequences of the ISM family. ISM appears to segregate with mycobacterial pathogenicity.

APPLICATIONS AND USES OF THE INVENTION

Cloned DNA probes of the invention from recombinant DNA libraries derived from specific mycobacteria or synthetic DNA sequences based on information from such libraries and selected for mycobacterial strain or phenotypic specificity, are used in the specific identification of mycobacterial DNA or RNA and the differentiation of mycobacterial from other microbial sequences. Such specific mycobacterial DNA probes are also used for the precise differentiation between mycobacterial strains and species and the recognition or prediction of phenotypic properties such as drug sensitivity and pathogenicity.

In addition, DNA or RNA probes or synthetic

oligonucleotides derived from all or part of the sequence ISM-1 (in pMB22 deposited with NCIB No.12451), ISM-2, and other members of the ISM family of mycobacterial insertion sequences of the invention sharing partial homology with ISM-1, are used as probes for the specific detection of potentially pathogenic strains of mycobacteria. These include *M. paratuberculosis*, pathogenic forms of *M. avium* including those involved in AIDS superinfections, and other pathogenic mycobacteria containing ISM sequences including *M. leprae*.

Precise identification and differentiation of mycobacteria as well as the specific detection of ISM-containing pathogenic strains, is applied to environmental samples such as water, animal and human food stuffs, and soil. Such applications are useful in the prevention and public health containment of mycobacterial diseases in humans and animals. Tests using the methods and probes of the invention are also applied to medical and veterinary samples of tissue, tissue extracts, body fluids, including milk, and excreta, as well as to the accelerated identification and characterisation of mycobacteria grown in in-vitro cultures, and to the improvement of such culture conditions. These applications are useful in the diagnosis of mycobacterial diseases such as Johne's disease in cattle and other animals, mycobacterial diseases in zoo animals and birds, human mycobacterial diseases such as leprosy, *M. avium* infections, AIDS mycobacterial superinfections, and to human Crohn's disease.

In methods used in the tests, DNA probes are single or double-stranded or are RNA transcripts, and may be unlabelled or labelled with radioactive, biotinyl, fluorescent, enzyme immunological or other revealing agents or methods. Probes representing all or part of ISM are used in solution phase systems or bound to solid phase supports such as cellulose, nylon, nitrocellulose, plastic, or gel systems, in the form of beads, tubes, or other matrix. Samples are treated to release mycobacterial DNA or RNA. Mechanical disruption, and/or sonication and/or enzymic treatments and/or heat treatment, and/or chemical treatments are used. Treated samples are mixed with the probe (boiled, if double-stranded) in a solution designed to allow hybridisation and prevent nuclease action. Hybridisation of specific probes to target DNA or RNA present in the sample is achieved by standard procedures.

Detection of hybrids is by a number of methods. Hydroxyapatite (HAP) may be added to bind double-stranded DNA or RNA, and the HAP is then washed to remove unhybridised probe. Other solid matrixes are used to bind hybrid. Alternatively, unhybridised probe is removed by digestion with single-strand specific nuclease followed by precipitation of hybrid by trichloroacetic acid, ethanol or other substance. Hybrids are detected by methods dependent on the revealing method used. Unlabelled probe DNA is bound to a solid matrix and used to hybridise to the sample. Target mycobacterial DNA or RNA homologous to the probe sequence becomes bound to the matrix via the probe. The matrix is washed and target DNA or RNA released by heating or transfer to denaturing solution. The target DNA or RNA is then

detected directly by the methods described above. Alternatively the DNA or RNA is first amplified by polymerase chain reactions (PCR [12]) using DNA polymerase or reverse transcriptase plus DNA polymerase together with specific oligonucleotide primers for sequences present in the probe or ISM sequence, and such primers form further aspects of the present invention. After amplification, target sequences are detected by any of the methods described above. Detection may involve differentiation between unreacted primers and primers that have been extended in the PCR reactions. In this case the primers are labelled and detection is dependent on the label used. Hybridisation of probe to unamplified or amplified target mycobacterial sequences is by standard procedures and dot blot methods or the identification of RFLPs are also used.

All or part of the mycobacterial insertion sequences of the invention including ISM-1 (in pMB22), ISM-2 and other members of the ISM family sharing partial sequence homology with ISM-1, are used to generate recombinant proteins or peptides or synthetic peptides based on ISM sequences and such derived sequences, form further aspects of the present invention. Also of use are proteins mutated or altered in expression by ISM.

Recombinant proteins and ISM derived peptides are also used in immuno and other diagnostic assays using standard techniques applicable to medical, veterinary, environmental and in-vitro culture samples for the specific detection of ISM-containing pathogenic mycobacteria. Antibodies to ISM derived proteins or peptides whether polyclonal or monoclonal for use in such diagnostic immunoassays or other systems form further aspects of the present invention. Recombinant proteins based on ISM sequences are used with or without computer-based structural studies for the rational design of specific drugs to modify or block their biological actions. Recombinant ISM proteins or peptides may themselves be used as therapeutic agents to mediate specific biological effects in vivo such as immunomodulation.

All or part of the DNA sequence of members of the ISM family may be used to engineer mutant bacteria or viruses and such mutant forms containing ISM sequences form further aspects of the present invention. These are employed as specific vaccines for the prevention of diseases caused by pathogenic mycobacteria in medical and veterinary practise. They may also be used as agents in immunotherapy or diagnostics. Proteins or peptides derived from ISM are also used as vaccines for the same purpose.

REFERENCES

1. Goodfellow M and Wayne LG. (1982). in *The Biology of Mycobacteria*. ed. Ratledge & Stanford. Academic Press. London.
2. Graham, DY, et al. (1987). *Gastroenterol* 92: 436-442.
3. Chiodini RJ, et al., (1984). *The Cornell Veterinarian* 74: 218-262.
4. Collins F. *Int.J.Leprosy* 54: 458-474.

5. Van Eden et al. (1988). *Nature* 331: 171-173.
6. Chiodini RJ et al. (1984) *Dig.Dis.Sci.* 29: 1073-1079.
7. McFadden JJ. et al. (1987) *J.Clin.Microbiol.* 25: 796-801.
8. McFadden JJ. et al. (1987). *Mol.microbiol.* 1: 283-291.
9. Collins J, et al. (1987). *Gastroenterol.* 92: 1352.
10. Martin CM, et al (1987). *Am.Rev.Res.Dis.* 136: 344-348.
11. McClure HM, et al. (1987). *J.Inf.Dis.* 155: 1011-1019.
12. Cullum J. (1985). in *Genetics of Bacteria*. ed.Scafe J. et al., Academic Press, London.
13. Scharf SJ, et al. (1987). *Science.* 233: 1076-78.

LIST OF FIGURES

Figure 1

DNA was extracted from Crohn's disease-derived strains: Ben (CD1), Linda (CD2) & Dominic (CD3); *M. paratuberculosis*, *M. avium* complex serotype 2, *M. avium* complex serotype 5, *M. kansasii*, & *M. phlei*. DNA samples (1µg) were digested with restriction endonuclease *PvuII*, electrophoresed through 1% agarose, blotted onto nylon membrane, hybridised to radiolabelled probe pMB22 and autoradiographed. Molecular weight markers, in kilobases are shown on the right.

Figure 2

DNA was extracted from Crohn's disease - derived strains 410 (obtained from G. Gitnick) and strain H1 (obtained from J. Haagsma); and *M. avium*. DNA samples (1µg) were digested with restriction endonuclease *PvuII*, electrophoresed through 1% agarose, blotted onto nylon membrane and hybridised to radiolabelled probe pMB22, and autoradiographed. Lane 1: *M. avium*, lane 2: strain H1, lane 3: strain 410.

Figure 3 A

Restriction endonuclease map of pMB22 showing location of sequencing contigs C1 and C2 (→) and also location of subclones S4, S8 and S12 (=====). The exact location of subclones has not been determined, therefore their maximum extent is indicated by hatched lines. P = *PvuII*, X = *XhoI*, R = *EcoRI*, B = *BglII*, K = *KpnI*. Molecular weight is indicated in kilobases (Kb).

Figure 3 B

Restriction endonuclease maps of the loci encoding sequences contained in pMB 22 in the genomes of *M. paratuberculosis* and *M. avium*. Molecular weight is shown in kilobases (Kb).

Figure 4

DNA sequence information obtained from the clone pMB22. Two contiguous lengths of sequence (contigs C1 and C2) are shown. The locations of the contigs are shown in the map of pMB22 in Fig 3A.

Figure 5

DNA was extracted from mycobactin-dependent disease-derived strains of *M. avium*, *M. paratuberculosis* and atypical *M. avium* strains. DNA samples (approx. 0.5µg) were digested with restriction endonuclease *PvuII*, electrophoresed through 1% agarose, blotted onto nylon membrane and hybridised to radiolabelled, pMB22 probe (A), pMB22/S4 probe (B) pMB22/S12 probe (C), and autoradiographed. DNA samples were: *M. paratuberculosis* (lane 1), non-mycobactin dependent *M. avium* serotype 2 (lane 2), non-mycobactin-dependent armadillo-derived strain 10911 (lane 3), mycobactin-dependent goat derived strain 8589 (lane 4), mycobactin-dependent armadillo-derived strains 157 (lane 5) and 157S (lane 6), mycobactin-dependent porcine-derived strain 7941 (lane 7), mycobactin-dependent deer derived strains 8446 (lane 8) and 8438 (lane 9), mycobactin-dependent wood-pigeon derived strains 1/79 (lane 10), 5/79 (lane 11), 1/72 (lane 12) and mycobactin-dependent goat-derived strain GK27, (lane 13). Molecular weight markers are shown on the right of A.

Claims

1. The plasmid pMB 22 containing ISM-1.
2. A host cell transformed by the plasmid pMB 22.
3. A DNA sequence from the genome of a mycobacterium which hybridises at low stringency with ISM-1.
4. A DNA sequence from the genome of a mycobacterium having at least 70% sequence homology with any contiguous stretch of at least 20 nucleotides within the ISM-1 of pMB 22.
5. A DNA sequence according to claim 4 having at least 70% sequence homology with the ISM-1 of pMB 22.
6. ISM-1.
7. A DNA or RNA probe comprising recombinant DNA or RNA, or synthetic oligonucleotides, based upon all or part of the DNA sequence according to any one of claims 3 to 6.
8. A DNA or RNA probe comprising recombinant DNA or RNA or synthetic oligonucleotides, based on DNA sequences mutated or otherwise altered in expression by the presence of a sequence according to any one of claims 3 to 6.
9. A DNA or RNA probe according to any one of claims 3 to 8, carrying a revealing label.
10. A bacterium or virus carrying a DNA or RNA sequence according to any one of claims 3 to 6 such that the DNA or RNA codes for a protein or peptide that is immunologically active.
11. A method for detecting the presence or absence of a mycobacterial strain or phenotype which comprises assaying a sample for mycobacterial DNA or RNA with a probe according to claim 9 or a bacterium or virus according to

claim 10.

12. A method for the precise identification and differentiation of mycobacterial strains, species or phenotypes by identification of restriction-fragment-length polymorphisms, using DNA probes.

13. A method according to claim 11 or 12 for the precise identification and differentiation of closely related mycobacterial strains and species.

14. A method according to claim 11, 12 or 13 wherein the or one of the mycobacterial strain(s) is M. paratuberculosis.

15. A method according to claim 11, 12 or 13 wherein the or one of the mycobacterial strain(s) is of the group M. avium-intracellulare or M. avium complex.

16. A method according to claim 11, 12 or 13 wherein the or one of the mycobacterial strain(s) is M. leprae.

17. A method according to claim 11, 12 or 13 wherein the or one of the mycobacteria cause AIDS superinfections or atypical tuberculosis.

18. A method according to claim 11 wherein the phenotype is pathogenicity or drug resistance.

19. A method according to claim 11, 12 or 13 wherein the mycobacterial strain causes Johne's disease in animals or Crohn's disease in humans.

20. A method according to any one of claims 11 to 19 wherein the sample comprises body fluids, tissue, tissue extracts, excreta, in vitro cultures, or environmental samples.

21. A method of detecting mycobacteria by a polymerase chain reaction enzyme amplification method wherein a DNA probe according to any one of claims 3 to 9 is used.

22. A recombinant protein or peptide encoded by all or part of the DNA or RNA according to any one of claims 3 to 9, or by the DNA or RNA of the bacterium or virus according to claim 10, or a synthetic protein or peptide of the same structure, the recombinant or synthetic protein or peptide optionally attached to one or more other proteins or peptides.

23. An antibody to any protein or peptide according to claim 22.

24. A vaccine against mycobacterial disease in animals or humans comprising bacteria or viruses according to claim 10, one or more recombinant or synthetic proteins or peptides according to claim 22 or antibody according to claim 23.

25. A pharmaceutical preparation comprising, as active ingredient, bacteria or viruses according to claim 10, one or more recombinant or synthetic proteins or peptides according to claim 22 or antibody according to claim 23.

26. An optionally labelled diagnostic reagent which comprises a DNA or RNA probe according to any one of claims 3 to 9, a bacterium or virus according to claim 10, a recombinant or synthetic protein or peptide according to claim 22 or an antibody according to claim 23.

27. An optionally labelled diagnostic reagent according to claim 26 immobilised on a solid phase,

28. A diagnostic test kit comprising, as one component, an optionally labelled reagent according to claim 26 or 27.

29. A diagnostic reagent according to claim 26 or 27 or a test kit according to claim 28 for the diagnosis of Crohn's disease in humans or Johne's disease in animals.

Fig. 1.

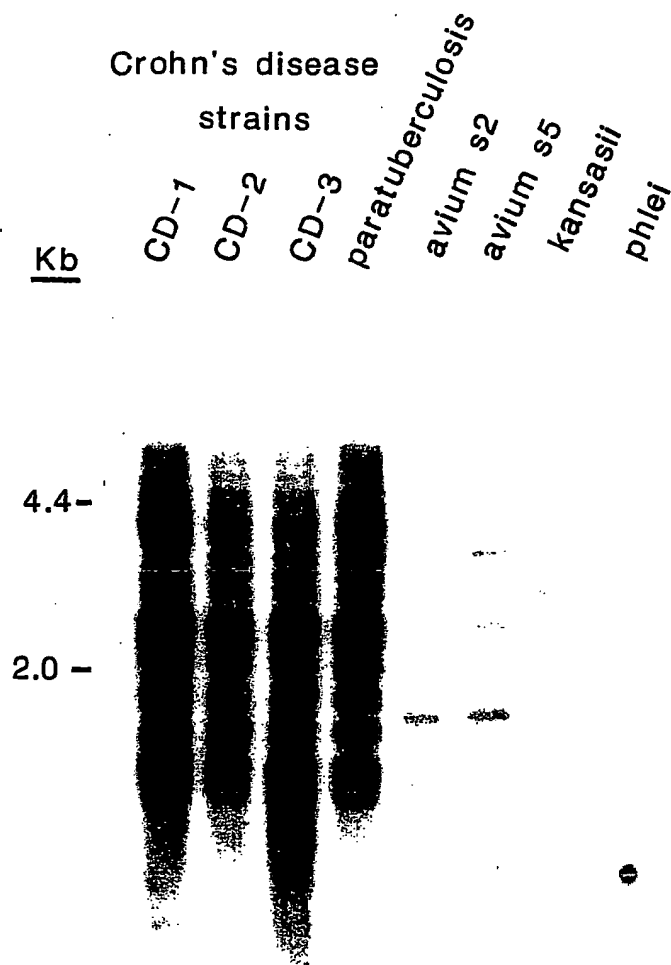


Fig. 2.

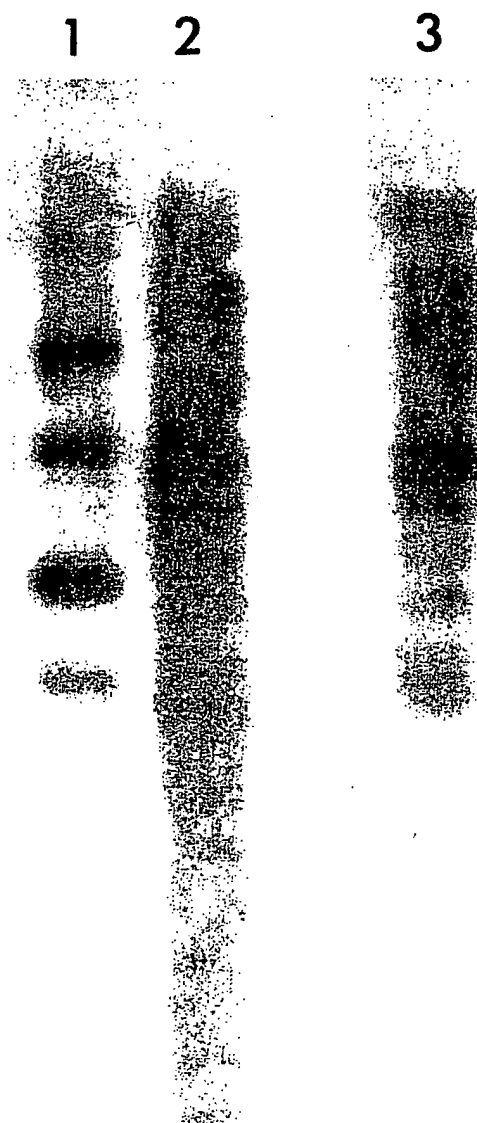


Fig. 3A.

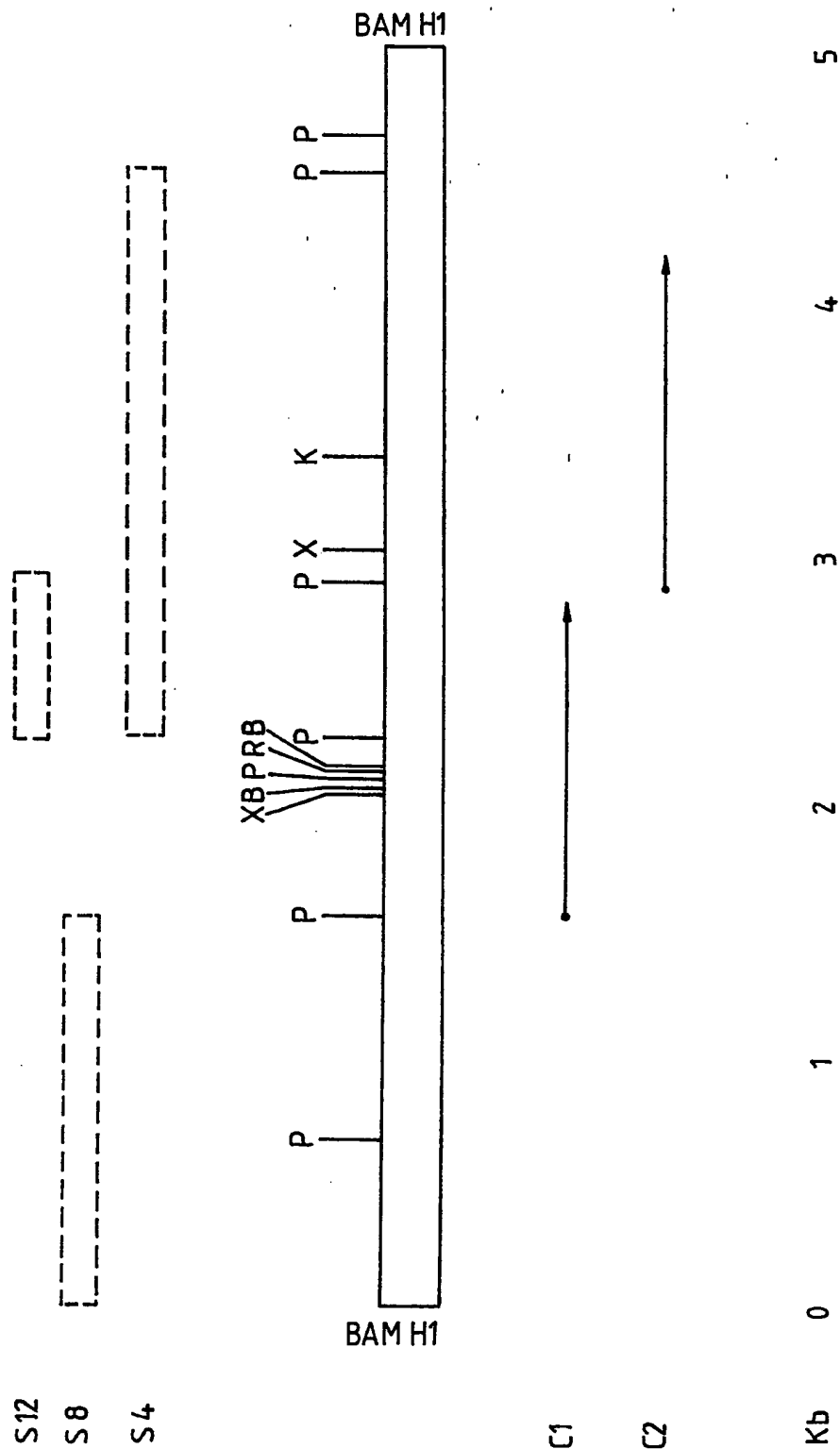
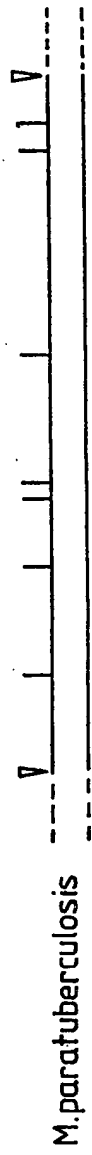


Fig. 3B.



Kb 0 1 2 3 4 5

BAMH1 V

PVU 2 I

Fig.4.

CONTIG 1

```

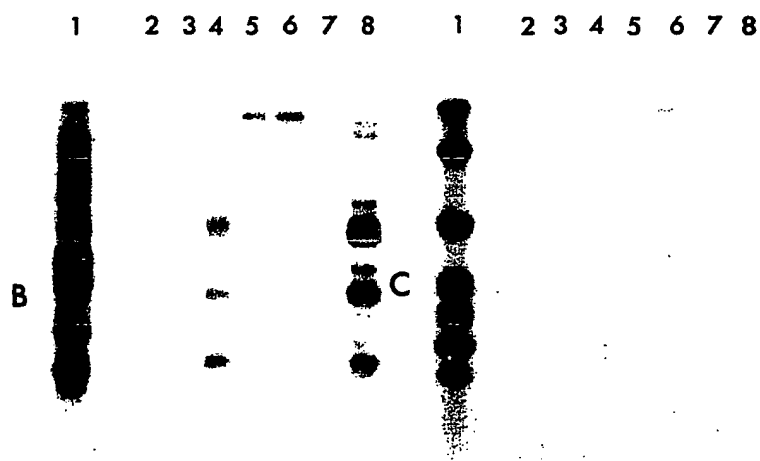
•CAGCTGGGTG  ACGGCTTCCC  ACACGTCGGG  CACCTCGTCC  AGCGGAACCC
ACACGCTGAG  CCGCTGGGCC  AGCATCGGGT  TGGTGGACAG  GCCGCCGCCC
ACCCACAGGT  CCAGGCCGGG  TCCGTGCTCG  GGGTGCTCGA  CGCCGACGAA
CGCGACGTCG  TGGGTCTCGT  GCGAGACGTC  CTGCAGGCCC  GAGACCGCGG
TTTTGTACTT  GCGCGGCAGG  TTGGCGTATT  CGGGGTGTGT  CAGGGAGCGA
CGGACGATCT  CCTCGATCGC  GGGCGACGGA  TCGAGCACCT  CGTCGAGCGA
GTGCCGGCCA  GCGGCGAGCC  GTGGATGCCG  CGCGGGCAGT  CCGCGCAGGC
CTCGGTGGTC  TGCAGCCCGA  CGGACTCGAG  CCGTCGCCAG  ATCTCGGGCA
CGTCCTCGAT  GCGAATCCAG  TGCAGCTGCA  GGTTTTCCCG  GTCGCTGATA
TCGGCGGTGT  CGCGGGCGAA  TTCGGTGGAG  ATCTGGCCGA  GCGTGCGCAT
GGTGTGCGCC  GACATCGCCT  TGCCGTGCGA  GCGGACCCGC  ATCATGAAGT
ACTTGGCCTC  GATCTTGTCG  GTGTTGTCTG  CACCGGTCCA  GCTGCCGTCT
TAGCCCTGCT  CGCGTTGGGT  GTACAGGCC  ATCCACCGGA  AGCGGCCGCG
CAGGTCGGAC  TTGTCGATGC  TGTCGAAACC  CTGCTTGGCG  TAGACGTTGA
TGATGCGCTC  GCATCGCCGA  TCTCGTTCAG  CGGCCCGTCG  GCCAGCTTGA
TCTTCTCGGT  GTCGTTGAGC  GGTTCGCGAT  CTCCCAGCGA  GCCCAGACCT
GACCCTCAGT  TGCGGGTCTT  GACGGGACGT  GCGGTGGTCA  TGTGGTGTTC
CTTACCTTTC  TTGAAGGGTG  TTCGGGGCCG  TCGCGCTTAG  GCTTCGAATT
GCCCAGGGAC  GTCGGGTATG  GCTTTCATGT  GGTGCTGTG  TTGGATGGCC
GAAGGAGATT  GGCCGCCCGC  GGCGTCCCGC  GACGACTCGA  CCGCTAATTG
AGAGATGCGA  TTGGATCGCT  GTGTAAGGAC  ACGTCGGCGT  GGTGCTCTGC
TGGGTTGATC  TGGACAATGA  CGGTTACGGA  GGTGGTTGTG  GCACAACCTG
TCTGGGCGGG  CGTGGACNCC  GGTAAGCGCG  ACCATTACTG  CATGGTTATT
AACGACGACC  GACNCGCGCA  CGATTGCTCT  CGCAGCGGGT  GGCCAACGAC
GAGGCCGCGC  TGCTGGAGTT  TGATTGCGGC  GGTGACGACG  TTGGCCGATG
GAGGCCGAGGT▶

```

*Fig. 4 (continued)*CONTIG 2

• GATCGACCTC ACAGCCGGCG GAGCCGGTTC GTGACTCGCT TCGTCCATGT
 CCGGGCAGCG GCTGCTTTAT ATTCCCAGGC GCACGGTCCA TCCAGCGGGT
 AGTTACCGCG CGGAAGGCAA GACCGACGCC AAAGACGCTG CGATCATCGC
 CGATCAGGCC CGGATGCGCC ACGACTTGCA GCCTCGTGCG CGCCGGCGAT
 GACATCGCAG TCGACGTGCG CATCCTGACC AGCCGACGTT CCGATCTGGT
 GGCTGATCGG ACCCGGGCGA TCAACCGAAT GCGCGCCCAG CTGCTGGAAT
 ACTTTTCGGCG CTGGAACGCG CCTTCGACTA CAACAAGAGC CGTGCCGCGC
 TGATCCTGCT TACTGGCTAC CAAACTCCCG ACGCGCTGCG TAGCGCCGGT
 GCGGCTCGAG TAGCCGCGTT CTTGCGTAAA CGAAGAGGGC CCGCAACGCC
 GATCACCGTC GCAGCCACCG CGCTGCAGGC CGCTAACGCC CAACACAGCA
 TCGTGCCCCG CCAACAACCTG GCGGCCACTG TGGTGGCCCCG CCTGGCCAAG
 GAGGTGATGG CCCTCGACAC CGAAATCGGC GACCACGACC GACGCGATGA
 TCGAGGAGCG ATTTTCGCGC CACCGCCACG CCGAAATTCA TCCTCAGCAT
 TGCCCGGATT CGGCGTCATC CTGGGCGCTG AGTTCCTCGC CACCGGCGGG
 GACATGGCCG CATTCGCCTC CGCCGACCGC CTCGGCGTCG CGGCCTGGCC
 TGGCGCCGGT ACCACGAGAT TCCGGCGCAT CAGCGGAAAC CTCAAACGCC
 CCCGACGCTA CGACCGGCGC CTGCTGCCGG CCTGCTACCT GTCGGCCTTG
 GTCAGCATCC GCACCGACCC CTCCTCGCGC ACCTACTACG ACCGAAAACG
 CACCGAAGGA AAACGCCACA CCCAAGCCGT CCTCGCCCTG GCCCGCCGCC
 GCCTCAACGT CCTGTGGGGC CATCGTGCGC GACCACGCTG TCTACCAACC
 CGCAACCACT ACCGCGGCGG CTTGACAACG TCATTGAGAA TCTCCTTCGC
 GAACATTCCC CGGTGTGCGA CGCACCGGGG TGGTGTTCCT GACGGCGGCT
 CAGATCGGCC TGCTACGGGG CCGGGATGGG CTGTGCGCCG CGGCATCGCG
 ATGATGTTGG GGCTTTAGGC ACCCGCAAGT TCGTCGTTGG ACCCACGGGT
 CGCGATTCCC ATAGTGCACT ACTTGCGGCC GCGTCAGTAG AGGTCGCATG
 TTGTGAGGGC ACAACCGGAC GTTGTGTGCC GCCGCTTAC GATGGGTGAC
 GCGGATCACT TTGCGGGCCA GGCGAATTGC CGGGCCGGTC GCTGCCGTGA
 GGTGGAAT TCCGGCCTGA TTGGCAACCT TGGTCAGGCC GGGCGTCAGA
 CCGAATCCCG TGGCGGAACG TCCGTTCTGC GGCCGCGGAG TTGGGCGGCG
 AGCGGGTGTA TCCGTGCAAT GTTGATCAGC GACCGCCGTT GCGGGCGGCG
 GT ►

Fig. 5.





European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 88 30 3657

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X,P	WO-A-8 800 009 (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) * Page 4, line 16 - page 5, line 16; page 13, line 13 - page 14, line 29; page 30, line 2 - page 34, line 21 * ---	1-29	C 12 Q 1/68 C 12 P 1/02 C 12 N 15/00 A 61 K 39/04
X,P	WO-A-8 800 977 (SYNERGEN INC.) * Page 2, line 16 - page 3, line 24; page 6, line 1 - page 7, line 3; page 8, table 1; page 24, line 7 - page 27, line 3; page 32, lines 2-34 * ---	1-29	
X	WO-A-8 600 019 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE) * Page 4, lines 6-20; page 15, line 1 - page 16, line 36 * ---	24,25	
X	WO-A-8 402 721 (GEN-PROBE PARTNERS) * Page 66, table 3; page 78, table 6III; page 93, lines 1-4; page 93, lines 21-24; page 99, line 17 - page 101, line 20 * ---	3-11,13 -20,26, 27	
X	EP-A-0 173 339 (ENZO BIOCHEM INC.) * Page 14, table I; page 37, line 2 - page 39, line 24 * ---	3-11,13 -20,26, 27	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 Q 1/00 C 12 P 21/00 C 12 N 15/00 A 61 K 39/00 C 12 R 1/00
Y	EP-A-0 079 139 (ORION CORP. LTD) * Page 5, lines 15-17; page 8, line 20 - page 9, line 6; page 26, line 1 - page 29, line 20 * ---	1-29	
Y	EP-A-0 196 215 (BIOGEN NV) * Page 2, line 23 - page 4, line 22; page 12, line 20 - page 14, line 11; page 24, line 4 - page 27, line 14; page 30, line 1 - page 34, line 12 * --- -/-	1-29	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 18-07-1988	Examiner VAN BOHEMEN C.G.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			

EPO FORM 1503 01.82 (P0401)



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
X,P	EP-A-0 229 442 (GEN-PROBE INC.) * Page 48, line 9 - page 49, line 8; page 51, example 42; page 51, example 43; page 52, example 44; page 56, line 1 - page 60, line 7 * ---	3-11,13 -20,26, 27	
X	EP-A-0 076 123 (J.A. WEBSTER Jr.) * Page 31, lines 2-21; page 56, lines 2-9 * ---	3-11,13 -20,26, 27	
X	EP-A-0 168 933 (S. ULITZUR) * Page 11, lines 10-30; page 13, lines 23-32; claims 1,2,18,26 * ---	3-11,13 -20,26, 27	
X	CHEMICAL ABSTRACTS, vol. 105, 1986, page 408, no. 222283v, Columbus, Ohio, US; J.T. CRAWFORD et al.: "Analysis of plasmids in Mycobacterium aviumintracellulare isolates from persons with acquired immunodeficiency syndrome" & AM. REV. RESPIR. DIS. 1986, 134(4), 659-61 * Abstract * ---	17	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
X	CHEMICAL ABSTRACTS, vol. 104, 1986, page 196, no. 63334y, Columbus, Ohio, US; A. LABIDI et al.: "Restriction endonuclease mapping and cloning of Mycobacterium fortuitum var. fortuitum plasmid pAL5000" & ANN. INST. PASTEUR/MICROBIOL., 1985, 136B(2), 209-15 * Abstract * --- -/-	12	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 18-07-1988	Examiner VAN BOHEMEN C.G.
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document			

EPO FORM 1503 03.82 (P0401)



European Patent
Office

EUROPEAN SEARCH REPORT

Page 3

Application Number

EP 88 30 3657

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
14	Y CHEMICAL ABSTRACTS, vol. 104, 1986, page 156, no. 1677d, Columbus, Ohio, US; A. LABIDI et al.: "Cloning and expression of mycobacterial plasmid DNA in Escherichia coli" & FEMS MICROBIOL. LETT., 1985, 30(1-2), 221-5 * Abstract * ---	1,3-9, 11-20, 26,27	
14	Y CHEMICAL ABSTRACTS, vol. 96, 1982, page 296, no. 65372x, Columbus, Ohio, US; J.T. CRAWFORD et al.: "Characterization of plasmids from strains of Mycobacterium avium-intracellulare" & REV. INFECT. DIS. 1981, 3(5), 949-52 * Abstract * ---	1,3-9, 11-20, 26,27	
14	Y CHEMICAL ABSTRACTS, vol. 103, 1985, page 175, no. 117200b, Columbus, Ohio, US; P.T. HAMILTON et al.: "Structure of genes and insertion element in the methane-producing archaebacterium Methanobrevibacter smithii" & MOL.GEN.GENET. 1985, 200(1), 47-59 * Abstract * -----	1,3-9, 11-20, 26,27	
The present search report has been drawn up for all claims			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Place of search	Date of completion of the search	Examiner	
THE HAGUE	18-07-1988	VAN BOHEMEN C.G.	
CATEGORY OF CITED DOCUMENTS		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document			

EPO FORM 150 01.82 (P0401)

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☒ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)